J. Biochem. **141,** 699–707 (2007)
doi:10.1093/jb/mvm075

Stable Supply of Large Amounts of Human Fab from the Inclusion Bodies in *E. coli*

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Received November 28, 2006; accepted February 25, 2007; published online March 23, 2007

Recombinant human Fab-H chain and L chain were separately expressed as inclusion body using *Escherichia coli*. After solubilization of Fab-H chain and L chain by the reduction and *S*-alkyldisulphidation in 8 M urea, about 100 mg of purified Fab-H chain and about 160 mg of L chain could be obtained from 1 l of each culture by ion-exchange chromatogram in the presence of 8 M urea. Combination of the lyophilized Fab-H chain and L chain could be efficiently folded to native human Fab by using the stepwise dialysis method and the human Fab was purified with cation-exchange chromatogram. In the folding procedure, it was found that cysteamine and cystamine with positive charge were effective to improve the folding yield of human Fab. Moreover, from comparison of folding yield in the presence of ten kinds of additives, it was suggested that taurine was effective to improve the folding of human Fab. Consequently, we could obtain about 60 mg of folded human Fab from 1 l of each culture under the optimum conditions.

Key words: folding, human Fab, rheumatoid factor, taurine.

Abbreviations: CTA, cystamine; CTE, cysteamine; DDP, 3,3'-dithiodipropionic acid; GSSG, oxidized glutathione; ME, mercaptoethanol; MP, mercaptopropionic acid; RF, rheumatoid factors; scFv, single-chain Fv.

Monoclonal antibodies have become a significant and growing fraction of all drug candidates (*1–6*). The developments of biological technologies have allowed the production of antibodies capable of recognizing a number of antigens. Moreover, antibody humanization technology have been developed and made in fully human form against essentially any target. CDR-grafting, transfer of xenogeneic CDRs into the framework of a human antibody, are well-established methods of humanized antibody (*7–9*). Phage display libraries from human germline sequences or B-cell Ig repertoires can provide human antibodies against numerous antigens (*10–12*). However, a critical factor in high cost of antibody-based drugs is that the cell culture production systems currently used for recombinant antibodies are not very efficient.

Small antibody fragments, such as single-chain Fv (scFv) or Fab, are now being considered for applications on various clinical diagnosis and therapy. These fragments can penetrate into a tissue more rapidly than intact antibody (*13*) and can be expressed in *Escherichia coli*, which would drastically reduce the cost associated with large-scale mammalian cell culture. Fab would be the most suitable fragments for clinical application because they are more stable than scFv fragments (*14*). Fab consists of V_H–C_{H1} (Fab-H chain) and V_L–C_L (L chain), in which the two polypeptide chains are linked covalently by a C-terminal disulphide bond, and remains the interaction of different domains. The interchain disulphide bond in the Fab plays an essential role in the stabilization of Fab (*14*). Each expression of recombinant Fab-H chain and L chain in *E. coli* can produce huge amounts of recombinant fragments as the inclusion body. There are few reports on the successful folding of murine Fab (*15*, *16*) or murine Fab' (*17*) directly from the denaturing inclusion body, which was produced by expressing each chain in *E. coli*. However, the folding system for stable supply of large amounts of human Fab fragments from the inclusion bodies is not sufficient to develop.

We previously developed a slow dialysis method for oxidative refolding from denatured and reduced state on intact murine IgG from hybridoma cells (*18*). In this article, we established the system for stable supply of large amounts of human Fab expressed from *E. coli* by the efficient folding using dialysis methods.

MATERIALS AND METHODS

Materials—RF gene was obtained from Dr I. Ezaki and Dr T. Watanabe in Medical Institute of Bioregulation, Kyushu University. SP-Toyopeal 650 M and DEAE-Toyopeal 650 M were purchased from Tosoh (Tokyo, Japan). 3-Trimethyl ammoniopropylmethanethiosulfonate Bromide (TAPS), rabbit IgG and alkalinephosphatase-goat anti-rabbit IgG antibody was purchased from Wako Pure Chemical (Osaka, Japan). All other chemicals used were of the highest quality commercially available.

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Construction of Expression Vector for Human Fab-H Chain and L Chain Gene—V_H gene and V_L gene were amplified by PCR on the expression vector encoding human rheumatoid factors (RF) scFv, which was previously constructed from human monoclonal RF (*19*). C_{H1} and C_L gene were obtained by PCR from human IgG_1 kappa gene and then these genes were connected to V_H and V_L gene by PCR, respectively. The amplified human Fab-H chain and L chain gene were inserted between NdeI and EcoRI sites of pET22b vector (Novagen, Madison, WI, USA) just downstream of the T7 promoter, respectively.

Expression Human Fab-H Chain and L Chain—The expression vector pET22b containing a gene for human Fab-H chain and L chain was introduced into *E. coli* BL21(DE3) codon plus RIL (Novagen, Madison, WI, USA), respectively. The transformant cell was grown at 37°C in 1 l of LB medium containing ampicillin of 50 μg/ml. The culture was allowed to grow until mid-log phase ($OD_{600} = 0.6–0.8$) and the expression of Fab fragment was incubated by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation for 4 h.

Purification of each Human Fab-H Chain and L Chain from Inclusion Body—The cultured cells were harvested by centrifugation for 8 min at 8,500 rpm. The pellets were suspended in 30 ml of 20 mM MOPS buffer, pH 7, and then sonicated 3 times for 5 min each times in an ice-water bath. The mixture was centrifuged for 15 min at 12,000 rpm. The precipitates were resuspended in 10 ml of 20 mM MOPS buffer, pH 7. After addition of DNase I (final concentration of 5 μg/ml) and RNase A (final concentration of 20 μg/ml), the solution was incubated at 37°C to decompose nucleic acids. Then sodium chloride was added to the solution until a final concentration of 0.8 M, and the mixture was centrifuged. The precipitates were suspended in 6 M guanidine solution (0.584 M Tris–HCl buffer, pH 8.6, containing 5.37 mM EDTA and 6 M guanidine hydrochloride) and reduced with 50 μl of mercaptoethanol (ME) at 40°C for 90 min under a nitrogen atmosphere. TAPS (225 mg), which reversibly reacts with the thiol groups to give protein of strong positive charge (*20*), was added, and the reduction solution was incubated at 40°C for 30 min to increase the solubility of the denatured Fab fragments. The reaction mixture was dialysed against 50 mM sodium acetate buffer, pH 5.5, containing 8 M urea and passed through an anion exchange (DEAE-Toyopeal) column (1.5 cm × 10 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.5, containing 8 M urea in order to remove the residues of nucleic acids. The flow-through solution was applied to a cation-exchange (SP-Toyopeal) column (1.5 cm × 90 cm) equilibrated with 50 mM glycine-HCl, pH 3.0, containing 8 M urea. The column was eluted with a gradient of 500 ml of equilibration buffer and 500 ml of the same buffer containing 1 M NaCl. The protein fraction was collected and lyophilized.

Folding Measurements of TAPS Fab-H Chain and TAPS L Chain—For the folding measurements, TAPS Fab-H chain and TAPS L chain was incubated at 25°C in 0.1 M Tris–HCl, pH 8.0, containing 1 mM EDTA, 3 mM ME, 1 mM oxidized glutathione (GSSG) and various concentrations (0.4, 1, 2, 3, 4, 5, 6, 7, 8 M) of urea and the protein concentration was 80 μg/ml. Using fluorescence spectroscopy, the folding of TAPS Fab-H chain and TAPS L chain was followed by monitoring a shift in emission maximum or in intensity at 330 nm or 350 nm for various concentrations of urea. Fluorescence intensity was measured with an F-2000 Fluorescence Spectrophotometer (HITACHI).

Folding and Purification of Human Fab—The folding of human Fab was carried out using the dialysis method according to the method of Maeda *et al*. (*18*) with slight modification. The lyophilized TAPS Fab-H chain and L chain were separately dissolved in 6 M guanidine solution. Each fragment solution were quantified by the absorption at 280 nm based on the amount of Trp and Tyr residues, respectively (*21*) and then were mixed in 1 : 1 molar amounts. ME (final concentration of 72 mM) was added in the mixed solution of fragments for the reduction. After the incubation at 40°C for 1.5 h, GSSG (final concentration of 26 mM) was added to the reduced solution (redox solution). The redox solution was diluted by dialysis buffer (0.1 M Tris–HCl buffer, 1 mM EDTA, pH 8.0 containing 8 M urea, 0.3 mM ME, and 0.1 mM GSSG) until 5 ml, and the diluted solution was dialysed against 100 ml of the dialysis buffer containing 8 M urea, 0.3 mM ME and 0.1 mM GSSG at 4°C for 4 h. Then, the stepwise dialysis was performed against 8, 4, 2, 1, and 0 M. The folding yields were evaluated from the peak area on ion-exchange HPLC against that of quantitated Fab which had been folded and isolated.

Binding Activity of Folded Human Fab—The binding activity of Fab was evaluated using a rabbit IgG by ELISA according to previous report (*19*). An ELISA plate was coated with 50 μl of folded Fab in 0.1 M sodium carbonate buffer, pH 9.6, at various concentrations (0.2–20 μg/ml) and overnight at 4°C. Blocking was done with 2% non-fat dry milk in 13.7 mM NaCl/0.27 mM KCl/0.43 mM Na_2HPO_4/0.14 mM KH_2PO_4, pH 7.3, PBS containing 0.05% Tween-20 (PBST). After washing with PBST, 50 μl of rabbit IgG as serially diluted with blocking buffer and as incubated overnight at 4°C. An alkalinephosphatase-goat anti-rabbit IgG was used for evaluating the binding activity of folded Fab. Colouring was obtained using a 1 mg/ml *p*-nitrophenyl phosphate substrate solution with 1 mM $MgCl_2$ in 0.1 M sodium carbonate buffer, pH 9.6, and absorbance at 405 nm of each well was recorded.

CD Spectrum of the Folded Fab—After the folded Fab was isolated by cation-exchange chromatogram, the protein solution was dialysed against 40 mM phosphate buffer, pH 7.0 and then the CD spectrum was obtained with a Jasco-J 720 spectropolarimeter.

RESULTS AND DISCUSSION

Expression and Purification of Human Fab-H Chain and L Chain—RF are autoantibodies that bind to the Fc region between C_{H2} and C_{H3} domains in human IgG (*22*, *23*). We previously reported that the scFv, which is a miniaturized antibody, was constructed from human RF. In this article, human Fab gene was constructed using human RF scFv. Human Fab-H chain and L chain were

successfully expressed in BL21(DE3) codon plus RIL, respectively. The expressions of both fragments were assessed by SDS–PAGE. (Fig. 1). These fragments were distributed as huge amounts of inclusion bodies. Inclusion body mainly contains peptidoglycans, lipids, nucleic acids, lipopolysaccharides and membrane proteins (*24*). The purification of inclusion bodies was necessary for the efficient folding of denatured protein (*25–28*) and the quantitative measurement of protein concentration in folding. Therefore, the purification of the fragments from inclusion bodies was performed according to the method described in our previous paper (*29*) with a slight modification. Since the theoretical pI of Fab-H chain is 9.1 and L chain is 6.7, it is expected that the introduction of positive charge to each fragment would increase the solubility under the condition where each fragment is purified. The cation-exchange chromatograms of TAPS Fab-H chain and TAPS L chain are shown in Fig. 2, respectively. Main peaks in Fig. 2 were identified to be purified Fab-H chain and L chain by SDS–PAGE, respectively (data not shown). From 1 l of each culture, about 100 mg of purified Fab-H chain and 160 mg of purified L chain were obtained.

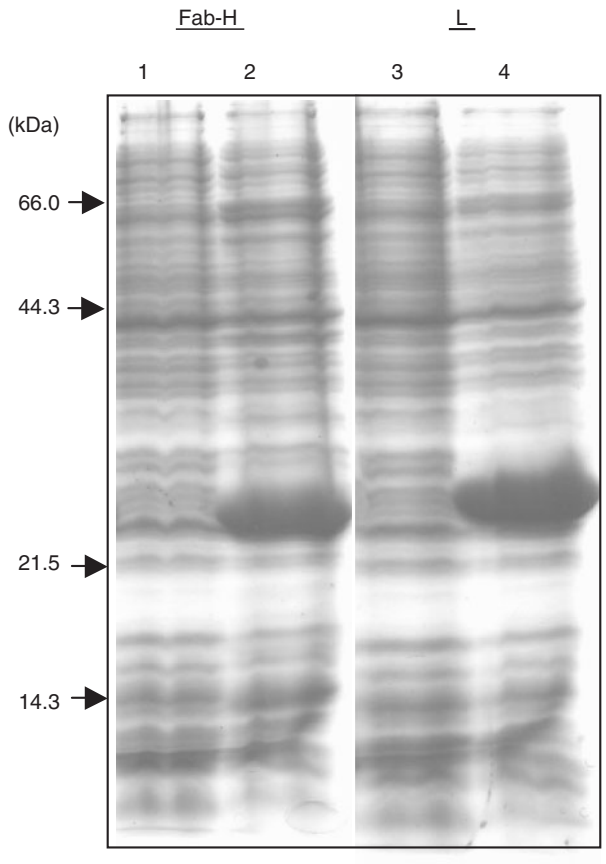

Fig. 1. **SDS–PAGE analysis of expression of human Fab-H chain and L chain from *E. coli*, respectively.** Lanes 1 and 2, the total extract *E. coli* cells harbouring pET22b/Fab-H chain gene, uninduced (lane 1) and induced with IPTG (lane 2). Lanes 3 and 4, the total extract *E. coli* cells harbouring pET22b/L chain gene, uninduced (lane 3) and induced with IPTG (lane 4).

Folding and Purification of Human Fab—Our previous study has shown that the refolding of murine IgG from denatured and reduced state are more effective than the dilution methods (*18*). Therefore, in this article, we attempted to fold the denatured and reduced human Fab using the dialysis method with slight modifications. Namely, the lyophilized TAPS Fab-H chain and TAPS L chain were separately dissolved in 6 M guanidine solution. Each fragment solution was mixed in 1 : 1 molar amounts. ME (final concentration of 72 mM) was excessively added against molar of fragments for reduction. After the incubation at 40°C for 1.5 h, GSSG (final concentration of 26 mM) was added to the reduced solution (redox solution). The redox solution was diluted by dialysis buffer (0.1 M Tris–HCl buffer, 1 mM EDTA, pH 8.0 containing 8 M urea, 0.3 mM ME and 0.1 mM GSSG) until 5 ml, and the diluted solution was dialysed against 100 ml of the dialysis buffer containing 8 M urea, 0.3 mM ME and 0.1 mM GSSG at 4°C for 4 h. In order to examine the suitable urea concentration of the folding of human Fab, the folding experiment of TAPS Fab-H chain and TAPS L chain against various urea concentrations was carried out using fluorescence spectroscopy, where an excitation wavelength is 280 nm and an emission wavelength is 330 and 350 nm. As shown in Fig. 3, the ratio of the fluorescence intensity (350 nm/330 nm) of both fragments did not change until 4 M urea concentration and then decreased from 4 M to 0 M urea concentration. These results indicate that the conformational change of both TAPS Fab-H chain and TAPS L chain drastically occur between 4 M and 0 M urea concentration. In the previous results, it was

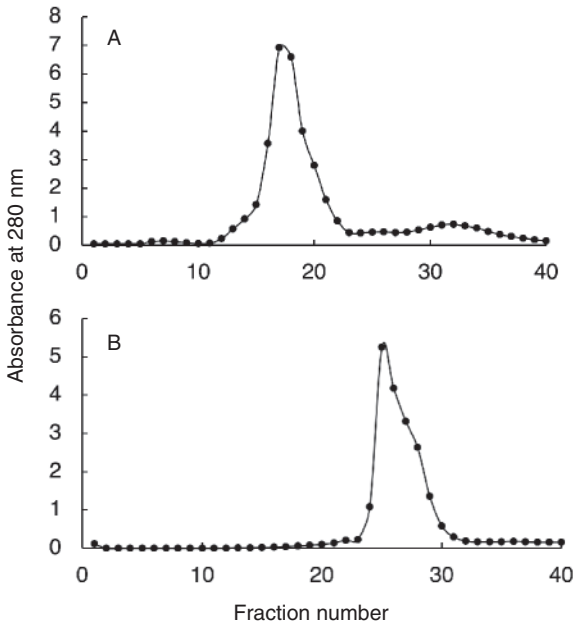

Fig. 2. **Cation-exchange chromatography of Fab-H chain (A) and L chain (B).** The column (SP-Toyopeal, 1.5 cm × 90 cm) was eluted with a gradient between 500 ml of 50 mM glycine containing 8 M urea, pH 3.0 and 500 ml of the same buffer containing 1 M NaCl.

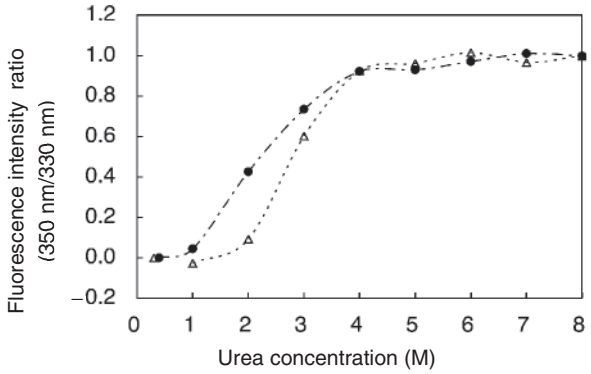

Fig. 3. **Fluorescence intensity ratio (350 nm/330 nm) of Fab-H chain (filled circles) or L chain (opened triangles) in the various concentrations of urea.** TAPS Fab-H chain and TAPS L chain was incubated at 25°C in 0.1 M Tris–HCl, pH 8.0, containing 1 mM EDTA, 3 mM ME, 1 mM GSSG and various concentrations (0.4, 1, 2, 3, 4, 5, 6, 7, 8 M) of urea and the protein concentration was 80 μg/ml.

suggested that the slow exclusion of denaturant from denatured protein during the occurrence of conformational change in the course of folding using dialysis was effective to form native conformation (*30*). Therefore, the stepwise dialysis on folding of human Fab was slowly performed between 4 M and 0 M urea concentration. The dialysis procedures of folding of human Fab are shown in Table 1.

At the first step, the lyophilized TAPS Fab-H chain and L chain were solubilized by 6 M guanidine solution due to increasing solubility of these lyophilized fragments against alkaline solution containing 8 M urea. However, the stepwise dialysis using guanidine instead of urea could not give the efficient folding of Fab (data not shown). Based on the results, in the oxidative folding of human Fab with interchain disulphide bond, urea would also be more effective than guanidine. On the other hand, at the final step, it took 100 h to dialyse against 0 M urea solution, although the other step was enough for 12 h. When the dialysis at 0 M urea solution was performed even for 24 h, the relative folding yield was 70% compared with that at the dialysis for 100 h. It was suggested that the dialysis in the final step was important for the oxidative folding of human Fab.

In order to isolate the folded Fab, we analysed the folded solution using ion-exchange chromatography. Cation-exchange chromatogram of the folding mixture of human Fab is shown in Fig. 4. The column (SP-Toyopeal) was eluted with a gradient between 50 ml of 50 mM sodium acetate buffer, pH 3.8, and 50 ml of the same buffer containing 0.5 M NaCl. SDS–PAGE of the protein in peak A showed that the protein band corresponding to Fab-H chain (23.6 kDa) or L chain (23.4 kDa) appeared under the reduced condition and that corresponding to folded Fab (47 kDa) appeared under the non-reduced condition (Fig. 5). From the amino acid analysis, the protein in peak A was identified to be the mixture of Fab-H chain and L chain, and the protein in peak B was identified to be L chain or L–L dimer (data not shown). Moreover, the binding activity

Table 1. **The dialysis step in folding of Fab.**

Step	Urea	Oxidant	Reductant	Dialysis time
1	8 M	3 mM	1 mM	4 h
2	8 M→4 M	3 mM	1 mM	4 h
3	4 M→2 M	3 mM	1 mM	12 h
4	2 M→1 M	3 mM	1 mM	12 h
5	1 M→0 M	...	...	100 h

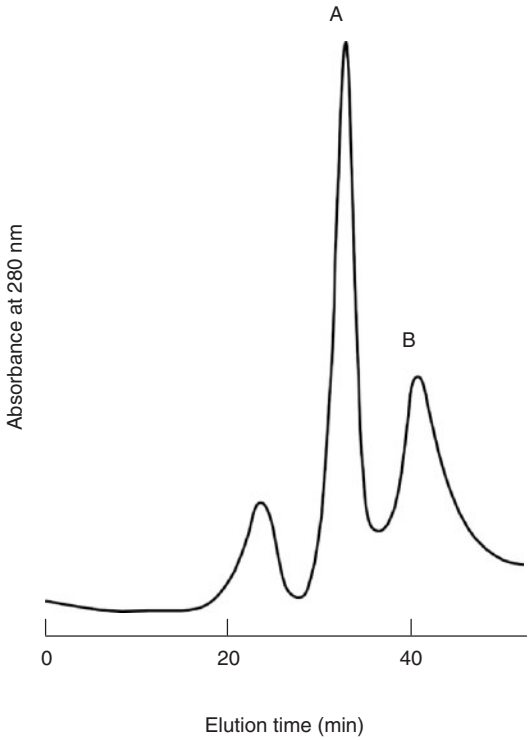

Fig. 4. **Ion-exchange HPLC of the folded solution of Fab.** The column (SP-Toyopeal, 0.8 cm × 52 cm) was eluted with a gradient between 50 ml of 50 mM NaOAc–AcOH, pH 3.8 and 50 ml of the same buffer containing 0.5 M NaCl at flow rate of 2 ml/min.

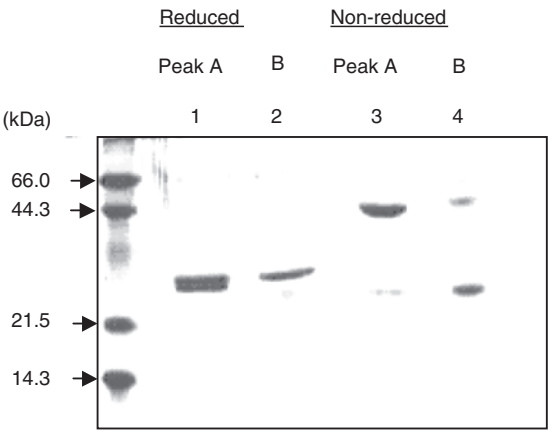

Fig. 5. **SDS–PAGE analysis of the proteins in peaks on the cation-exchange HPLC of folded solution of Fab.** Lanes 1 and 2, under reducing condition. Lanes 3 and 4, under non-reducing condition.

of the protein in peak A was measured by ELISA. It was known that human RF crossreacted to the rabbit IgG (*19*, *31*). As shown in Fig. 6, the amount of protein in peak A bound to the rabbit IgG increases with an increases of the protein concentration. The CD spectrum of the protein in peak A was measured (Fig. 7). The spectrum indicated the typical beta sheet and was consistent with that of intact Fab measured by Tetin *et al*. (*32*). These results indicated that most protein in the peak A have a native conformation. Thus, we defined the protein in the peak A as the correctly folded Fab and could be isolated in cation-exchange chromatogram (as peak A in Fig. 4).

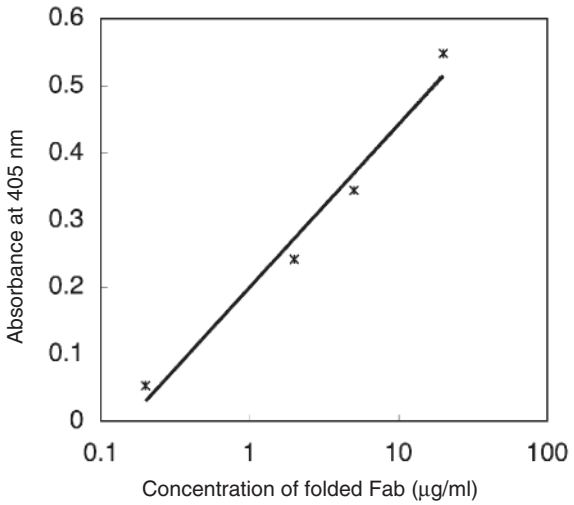

Fig. 6. **Binding activity of folded Fab against rabbit IgG.** Folded Fabs with various concentrations were coated on the ELISA plate, followed by addition of serially diluted rabbit IgG.

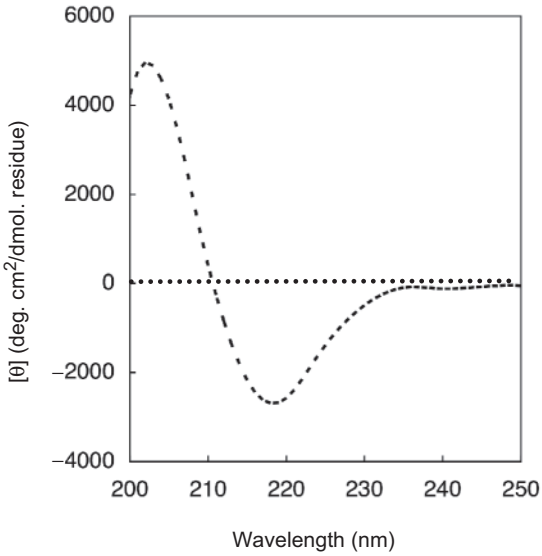

Fig. 7. **CD spectrum of folded Fab.** The CD spectrum was obtained with a Jasco-J 720 spectropolarimeter.

The protein concentration in the folding of oligomeric protein is a very important factor. At a low protein concentration, the subunits would be hard to interact each other, and at a high protein concentration, unfavourable aggregation between subunits would often occur (*18*, *33*, *34*). In order to investigate the effective condition of folding, the folding yields of Fab at the several concentrations of lyophilized Fab-H chain and L chain were determined from peak area of the folded Fab in cation-exchange chromatogram. The relative folding ratio at various protein concentrations against the maximum folding yield is shown in Fig. 8A. The protein concentration for maximum ratio was about 0.2 mg/ml, where the folding yield was 24%. The folding yield gradually decreased with an increase in protein concentration. On the other hand, in Fig. 8B, the relative recovery ratio of the folded Fab at various protein concentrations is shown. The recovery amount of the folded Fab obtained from 1 ml of dialysis solution on the folding process was maximum at concentration of 1.2–1.6 mg/ml. These results were consistent with the observed dependence of protein concentration on the folding of murine Fab' (*17*). Thus, the interaction of Fab-H chain might be likely to occur the aggregation in the folding process from denatured Fab-H chain and L chain. The following experiments of the folding of

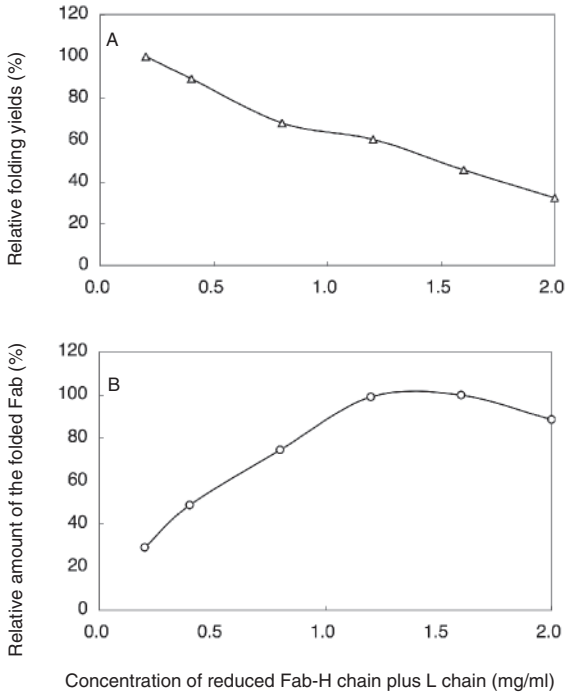

Fig. 8. **(A) Dependency of folding yields of Fab against various protein concentrations.** The longitudinal axe (relative folding yields) indicates the relative ratio (the ratio of the folding yields at various protein concentrations to the maximum folding yield). (B) The recovery of the folded Fab. The longitudinal axe (relative amount of the folded Fab) indicates the relative ratio (the ratio of the amount of folded Fab obtained from 1 ml of the dialysed solution to the maximum amount of folded Fab).

human Fab were performed at the concentration of 0.8 mg/ml.

Effect of Oxidizing and Reducing Reagents on the Folding of Human Fab—In our previous study, it was demonstrated that the charge on the oxidizing reagents affected the folding of reduced lysozyme (*35*). In order to examine whether the folding yields of human Fab was improved by the charge on the oxidizing and reducing reagents, positive charge reagents: cysteamine (CTE) and cystamine (CTA) and negative charge reagents: mercaptopropionic acid (MP) and dithiodipropionic acid (DDP) were used at the concentration of 0.3 mM (red) and 0.1 mM (oxi) in the folding of human Fab, as the replacement for ME and GSSG in the above methods. In the reduction reaction at initial stage on the folding experiment, the reducing reagents were excessively added against concentration of TAPS Fab-H or TAPS L chain in order to dissociate TAPS from proteins. Then, TAPS was sufficiently diluted at the initial dialysis step. Therefore, it was considered that TAPS has little effect for folding process. As shown in Fig. 9, the positive charge reagents could increase the folding yields about 1.5 folds, as compared with the folding yields at the reagents, ME and GSSG. On the other hand, the folding yields with the negative charge reagents significantly decreased (Fig. 9). Thus, it was found that the charge on the oxidizing and reducing reagents also affect the folding of human Fab. In the case of lysozyme, which is a basic protein, the introduction of positive charges derived from oxidizing reagent to reduced lysozyme through disulphide bonds in the early stage of folding, resulted in the increase of the solubility that, inturn, led to the depression of aggregation (*35*). This was consistent with the present case. Namely, because the theoretical pI of human Fab is 8.6, the introduction of positive charge to human Fab via disulphide bonds increases the solubility under the condition, resulting in the improvement of the folding yield of human Fab.

It is also known that the concentration or ratio of oxidizing and reducing reagents affect the oxidative folding (*15*, *36*, *37*). Using CTE and CTA, the effect of the concentration of reducing and oxidizing reagents on the folding yield of human Fab was examined (Fig. 10). In comparison with the condition where the concentration of reducing and oxidizing reagents were 3 mM CTE and 1 mM CTA or 0.03 mM CTE and 0.01 mM CTA, the folding of human Fab at the concentration of 0.3 mM CTE and 0.1 mM CTA was efficient. These results indicated that the reducing and oxidizing reagents at the low concentration were unlikely to facilitate sulphydryl-disulphide exchange reaction on the folding of human Fab and these reagents at the high concentration would prevent the efficient sulphydryl-disulphide exchange reaction. In the ratio of the reducing and oxidizing reagents, it was found that the ratio of 1:1 CTE–CTA was the optimum condition on the folding of human Fab.

Moreover, we prepared Fab-H chain and L chain lacked C-terminal cysteine by gene engineering, respectively, and folded these fragments by using the stepwise dialysis method as described above. The folding yield

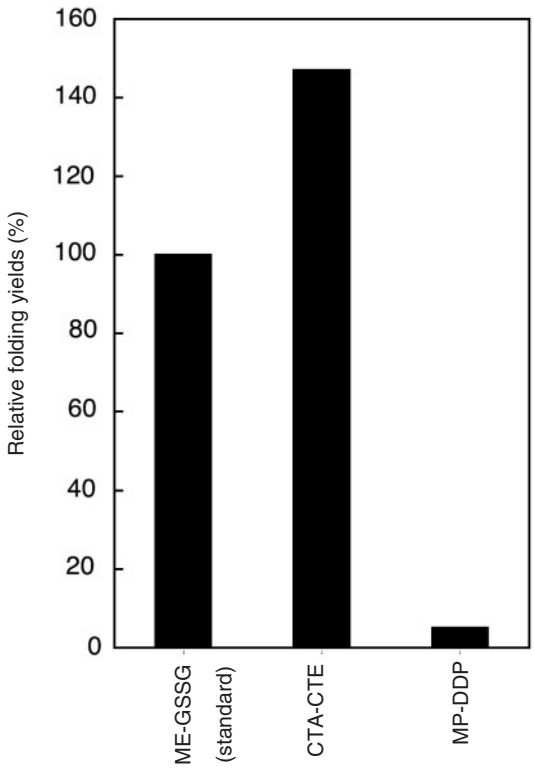

Fig. 9. **Effect of the reducing reagent and the oxidizing reagent in the folding buffer on the folding yields of Fab at the concentration of 0.3 mM (red) and 0.1 mM (oxi).** The folding yields of Fab are expressed relative to that in the presence of 0.3 mM CTE and 0.1 mM CTA. ME–GSSG; mercaptoethanol–oxidized glutathione. CTE–CTA; cysteamine–cystamine. MP–DDP; mercaptopropionic acid–3,3'-dithiodipropionic acid.

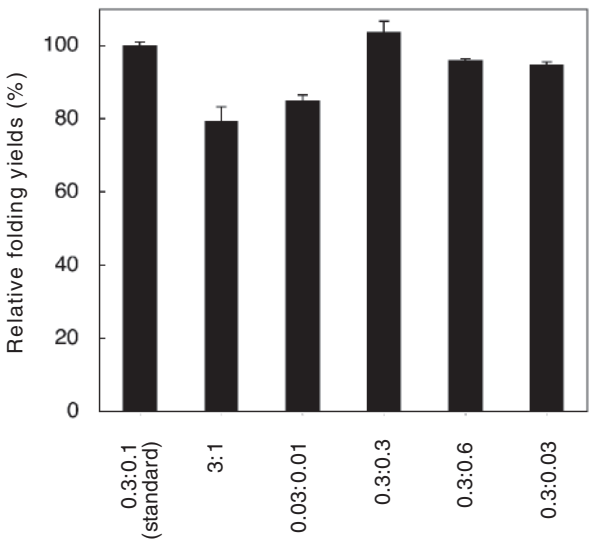

Fig. 10. **Effect of the concentration of CTE (0.03, 0.3, 3 mM) and CTA (0.01, 0.03, 0.3, 0.1, 0.6 mM) in the folding buffer on the relative folding yields of Fab.** The folding yields of Fab are expressed relative to that in the presence of 0.3 mM CTE and 0.1 mM CTA.

of the Fab without interchain disulphide bond was almost the same as Fab with interchain disulphide bond (data not shown). Namely, the existence of interchain disulphide bond was not critically important for the folding of Fab. If the formation of interchain disulphide bond in the folding process is involved in the association between Fab-H chain and L chain, the aggregation of Fab fragments are suppressed resulting that the folding yield of Fab would be increased. From these results, it was suggested that the formation of the disulphide bond is not involved in the conformational transition of the folding at least under this condition. The formation of intrachain disulphide bonds to stabilize the folded conformation in each chain would be rather important for the folding process of Fab.

Effect of Additive on the Folding of Human Fab—It have been reported that some additives can enhance the folding yields (*38–41*). In order to examine the effect of additive, human Fab was folded in the presence of 10 different kinds of additives as shown in Fig. 11. Each additive was added in the dialysis solution from the first step to the final step as shown in Table 1. Cation-exchange chromatogram of the folding mixture of Fab in the presence of taurine is shown in Fig. 12. The chromatograms of that in the presence of other additives also had patterns similar to that in the absence of additive as shown in Fig. 4. In Fig. 13, the relative folding yields of human Fab for 10 additives are shown as compared with the non-additive condition.

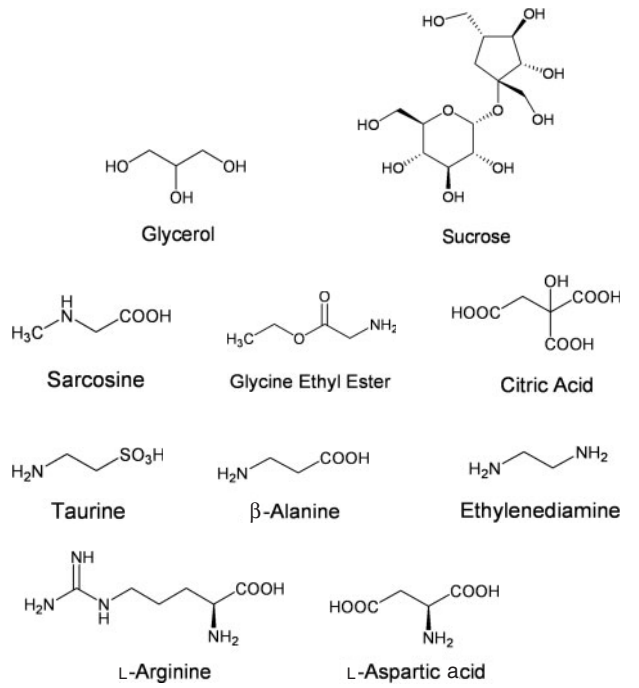

Fig. 11. **Chemical formulas of the additive agents used in the folding of Fab.**

Table 2. **The folding yield of human Fab in this study.**

Oxidizing and reducing reagents	Relative folding yields (%)	Amounts of Fab from each 1 l culture cells of Fab-H and L chain in *E. coli*
ME–GSSG	100	33.6 mg
CTE–CTA	147	48.9 mg
CTE–CTA with taurine	182	60.5 mg

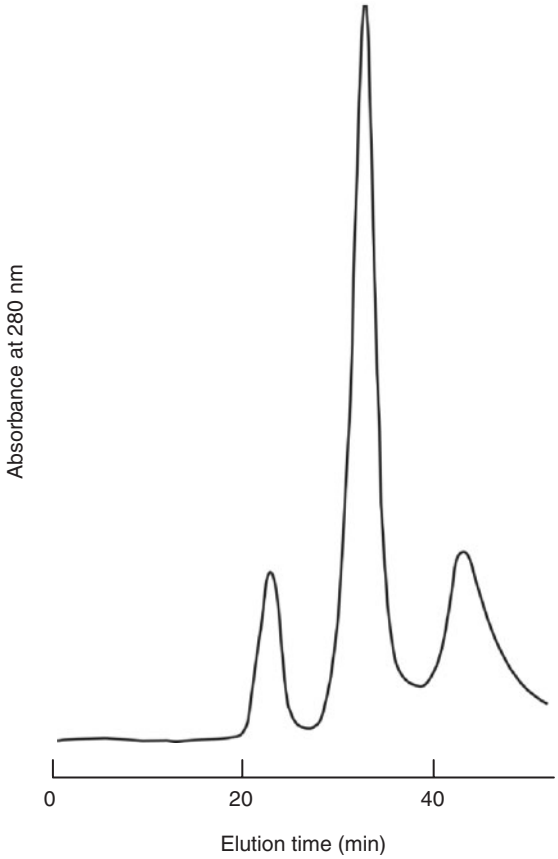

Fig. 12. **Ion-exchange HPLC of the folding mixture of Fab in the presence of taurine.** The column (SP-Toyopeal, 0.8 cm × 52 cm) was eluted with a gradient between 50 ml of 50 mM NaOAc–AcOH, pH 3.8 and 50 ml of the same buffer containing 0.5 M NaCl at flow rate of 2 ml/min.

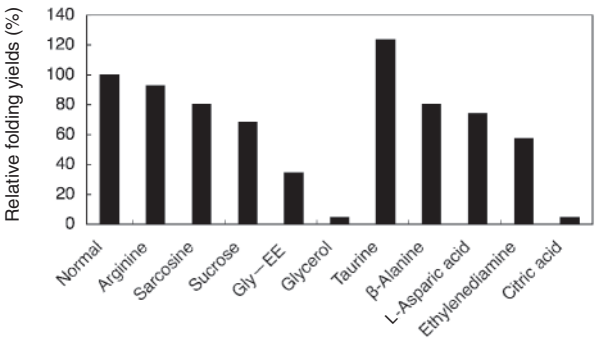

Fig. 13. **Effect of the additive agents in the folding buffer on the folding yields of Fab.** The folding yields of Fab in the presence of various additives are expressed relative to that in the absence of additive.

Arginine has been reported to be an efficient additive for the folding of Fab (*15*). However, in the present case, the folding yield of human Fab was not enhanced with arginine, whereas the aggregation on the folding of human Fab was apparently prevented. This was supported by the result that arginine did not facilitate refolding, but suppress aggregation of the proteins during the folding (*42*). Therefore, arginine is not always efficient additive for the folding of all Fabs, particularly in the human Fab.

The relative folding yields of the human Fab could not enhance in the presence of glycerol, sucrose and glycine ethyl ester, although these are efficient additives for other proteins (*43–48*). On the other hand, taurine is only effective for the folding, since the folding yield with taurine increase by 1.2-folds than that without the additive (Fig. 13). There is no report that taurine is effective to improve protein folding. Moreover, in order to understand why the folding yield increases in the presence of taurine, human Fab was folded in the presence of taurine analogues (Fig. 11). For example, beta-alanine and ethylenediamine have structures similar to taurine with the exception of the sulphonyl group (Fig. 11). Since the relative folding yields in the presence of those two additives decreased, the sulphonyl group of taurine may be involved in the improvement of the folding process of human Fab.

Taurine is one of osmolytes that are small molecules used in cells to protect organisms against stress of high osmotic pressure (*49*, *50*). Many osmolytes have been found to stabilize the native state of proteins relative to the unfolded one (*49–52*), but it is still unknown the effect of taurine on the stabilization of protein. Ratnaparkhi's studies showed that taurine stabilizes RNase A but destabilizes RNase S and S pro, whereas sarcosine stabilizes all three proteins (*49*). Based on these results, taurine might affect on denatured state of a protein. Anyway, in this article, we found that taurine was efficient additive for the folding of human Fab.

Finally, our results of the folding of human Fab from denatured and reduced form are shown in Table 2. We could obtain about 60 mg of folded human Fab from 1 l of each culture under the optimum conditions. The present procedure would help to provide a stable supply of large amounts of human Fab.

CONCLUSIONS

In this article, we established the procedure for stable supply of large amounts of human Fab expressed from *E. coli* by the efficient folding using dialysis methods. By ion-exchange chromatogram in the presence of 8 M urea of the reduced and *S*-alkyldisulphidation of Fab fragment with TAPS, the huge of Fab-H and L chain was purified. Moreover, the folding yields of human Fab could be improved by using both oxidizing and reducing reagents with positive charge, CTE and CTA, and taurine as the additive reagent.

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